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Molecular detection and genotyping of human respiratory viruses

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MOLECULAR DETECTION AND GENOTYPING OF HUMAN RESPIRATORY VIRUSES

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ABSTRACT
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HUMAN RESPIRATORY VIRUSES

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This study describes a reverse transcription polymerase chain reaction (RT-PCR) assay targeting the 5'noncoding region (5'NCR) for genotyping human rhinoviruses (HRVs). Phylogenetic relationships among prototype strains were determined based on sequences within the 5'NCR. Sequence identity comparisons between clinical isolates and prototype strains were used to genotype clinical isolates. All HRV clinical isolates associated with a specific HRV prototype strain, except for one isolate which may represent a novel HRV. Results indicate that the 5'NCR provides a sensitive and specific target for rapidly genotyping HRVs.

Additionally, preliminary studies were performed to determine the feasibility of microarray technology for simultaneous detection and genotyping of HRVs. Microarrays containing RT-PCR products from 5'NCR and VP4/VP2 regions of various HRVs were hybridized with Cy3 - labeled cDNA from known strains. Unique and selective hybridization patterns were seen for each tested strain. Microarray technology may provide a high throughput platform for detecting and genotyping HRVs.

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PREFACE

This thesis consists of three chapters and the appendixes. Each chapter is followed by a list of references cited for that particular chapter. Chapter I is a detailed introduction of the human rhinovirus, clinical manifestations of human rhinovirus infections, methods available for virus identification and typing, and the goals of the research. Chapter II and Chapter III are presented in manuscript format consistent with the Journal of Clinical Microbiology. Chapter II is a manuscript reporting the use of the reverse-transcription polymerase chain reaction assay based on the 5' non-coding region for detection and genotyping of human rhinoviruses, to be submitted for journal peer review. Chapter III is a manuscript reporting the use of microarray technology for simultaneous detection and genotyping of human rhinoviruses, also to be submitted for journal peer review. The appendixes provide additional references, as well as, additional details about the data collected and the equipment used for this research.

CHAPTER I

INTRODUCTION

Human Rhinovirus

Human rhinoviruses (HRV), the most frequent etiological agents of the common cold, belong to the *Picornaviridae* family (35). HRVs are non-enveloped, icosahedral, single-stranded, positive-polarity RNA viruses with a genome of approximately 7.1 kb. The genome includes a terminal VPg protein covalently attached to the 5' non-coding region (5'NCR) that is approximately 600 nucleotides (nt) long. A coding region, approximately 6500 nt long, follows the 5'NCR and includes genes which encode for viral capsid proteins and enzymes involved in polyprotein processing and genome replication. The coding region is followed by a short 3' non-coding region (3'NCR) (approximately 100 nt) and a polyA tail of about a dozen nucleotides (1) (Figure 1).

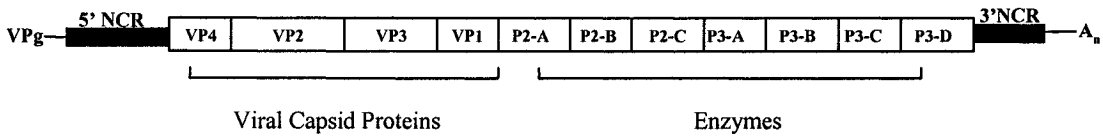

Figure 1. A schematic representation of HRV genome.

The HRV genome is packaged within a non-enveloped, icosahedral capsid consisting of 60 protomers arranged along a fivefold axis. Each of the protomers is composed of four viral structural proteins - VP1, VP2, VP3 which are exposed on the capsid surface, and VP4 which forms an interface between the capsid and internal genome (34). There are greater than 100 distinct serotypes of HRV that are divided into two groups based on host-cell receptor specificity. Major receptor group HRVs infect human respiratory epithelial cells using the intracellular adhesion molecule (ICAM)

receptor (25, 40). Minor receptor group HRVs utilize low density lipoprotein receptors (LDLR) to infect host cells (19). Phylogenetic studies based on the analysis of VP4/VP2 region of all prototype strains further divides human rhinoviruses into two genetic groups, HRV-A (consisting of 76 serotypes) and HRV-B (consisting of 25 serotypes) (36). Only HRV type 87 clusters individually and further characterization has revealed that it contains features of both HRV and the closely related human enterovirus (HEV) type 68, therefore, is classified as HEV-D (5). Major receptor group and minor receptor group HRVs are distributed among HRV-A and HRV-B.

Clinical Manifestations of Human Rhinovirus

Human rhinoviruses have long been known to cause self-limiting, acute upper respiratory illnesses such as the "common cold" (8, 16). Common colds due to rhinovirus are usually widespread during spring and autumn (3, 14). Transmission of the virus is facilitated via direct hand-to-hand contact and aerosolization, resulting in person-to-person spread (17). Rhinorrhea, nasal stuffiness, sore throat, fever, and general malaise are characteristic symptoms of the common cold (35). On average, children experience 5 to 7 HRV infections per year, whereas adults experience 2 to 3 HRV infections per year (35). Immunity to rhinovirus is serotype-specific, therefore protection against one serotype may not prevent infections by other serotypes (15).

Although a rhinovirus cold may seem mildly inconvenient to a healthy individual, with symptoms lasting a few days, HRV infections may result in more severe symptoms in infants, elderly, and immunocompromised patients (4). HRVs have been associated with pneumonia, sinusitis, otitis media, and exacerbations of asthma and chronic

obstructive pulmonary disease in high risk populations (2, 13, 14, 18, 22, 32). Early childhood rhinovirus infections have also been linked to an increased risk of childhood asthma and allergic diseases (28). Recent HRV outbreaks in long term care facilities for elderly persons have been associated with unusually high morbidity and high mortality (18, 30, 43). Mechanisms behind these observed associations are not yet clearly understood, but there is increasing evidence that rhinoviruses may illicit cellular responses that cause injury to the respiratory epithelium (7, 9, 23).

Virus Identification and Subtyping

Since acute respiratory illnesses can be caused by myriad viruses or bacteria, clinical diagnosis based solely on patient symptoms is ineffective in determining etiology. Characterization of illnesses caused by HRV is further hindered due to a lack of an animal model. Traditional laboratory diagnosis of HRV is accomplished by observing characteristic cytopathic effects (CPE) *in vitro* cell culture (12). Infection of a confluent monolayer of human fibroblasts with HRV causes CPE to appear within a week, but can take up to two weeks. CPE due to HRV infection of human fibroblasts are characterized by increase in cell granularity, rounding up of cells, cell lysis, and destruction of cell monolayer.

Neutralization assays using type-specific neutralizing antibodies from immune sera can facilitate detection, as well as, strain-typing of the virus (24). Despite being the current "gold standard", cell culture based assays can be time-consuming and tedious due to the great number of HRV serotypes. Furthermore, intact virus particles may not be present in all collected clinical specimens, therefore, cell culture based assays requiring *in*

vitro replication of the virus may not be highly sensitive. Also, immune sera are in limited supply and usually restricted to research laboratories, hindering the serotyping of human rhinoviruses in routine clinical settings.

Lack of rapid, specific, and sensitive assays that can not only detect, but differentiate between rhinovirus strains, is one of the major obstacles in correlating specific rhinovirus strains with more severe symptoms. Molecular based assays are relatively inexpensive, timely, and efficient tools for characterizing HRV (20, 42). Previous molecular assays based on 3DPol, VP1, 2A, and VP4/VP2 regions have been used to characterize HRV genus phylogeny (26, 27, 36, 37). Limited evaluation of the VP4/VP2 region has been performed for genotyping of HRV clinical isolates (38). However, these regions display varying degrees of conservation and may not be able to detect variant strains or provide resolution of closely related strains of HRV.

The VP4/VP2 region is currently used at the California Department of Health Services, Viral and Rickettsial Disease Laboratory as a target for genotyping HRV clinical isolates using a reverse-transcription polymerase chain reaction (RT-PCR), as previously described (38). Although there was a high correlation between VP4/VP2 RT-PCR results and traditional microneutralization assay, a certain percentage of the clinical isolates were not detected using the molecular assay. Therefore, another candidate region that was more conserved, more specific and would possibly increase the sensitivity of the RT-PCR based assay for detecting and genotyping HRV clinical isolates was considered.

The 5'NCR was considered as a target because this region contains *cis* - elements that are involved in viral replication and translation of viral RNA and is conserved

throughout the *Picornaviridae* family. Within the 5'NCR is the internal ribosomal entry site (IRES), which is used by the virus to translate its genome in a cap-independent method. The IRES has distinct clover-leaf like structural features that is different for different picornaviruses, thus can be used to distinguish HRVs from other picornaviruses (6, 33, 46).

Research Goals

The first goal of this research was to develop a reverse transcription polymerase chain reaction (RT-PCR) based molecular assay focused on approximately 400 nt region within the 5'NCR in order to detect and genotype HRVs in clinical samples. As with a previous study within the VP4/VP2 region (36), genomic sequences in the 5'NCR can be used to assess phylogenetic relationships among the prototype strains. Consequently, sequence homology comparisons between the prototype strains and clinically isolated strains can be efficiently used to genotype clinical isolates (38). This approach to genotyping can be used to collect meaningful epidemiological data that can identify circulating HRV strains involved in outbreaks and/or severe respiratory illnesses in populations at risk. Further analysis of such data should provide detailed information about specific human rhinovirus serotypes associated with more severe respiratory symptoms. Besides providing a more comprehensive understanding of the specific HRV serotypes that are involved in outbreaks, asthma exacerbations, allergies, and more severe symptoms, such data may offer insights into novel treatments and therapies for HRV infections and improve patient care via rapid diagnostics.

Secondly, we sought to develop a cDNA microarray for higher throughput detection and identification of human rhinoviruses. Microarray technology makes use of precision robotics to immobilize nucleic acids, organized as microscopic arrays, on glass slides, silicon chips, or nylon membranes (11). Further, microarrays are sensitive, require small sample volumes, and results from microarray analysis are statistically significant. Using sequence specific hybridization of nucleic acids, microarrays can be used to study genomics, targets for drug discovery, candidates for vaccine development, and evolution (10, 21, 39, 45). Such technology can also be used in novel ways to detect as well as subtype viruses (29, 31, 41, 44). Microarray based diagnostics can eliminate the need for PCR amplification and sequencing for genotyping clinical isolates, leading to rapid, sensitive, and specific viral diagnostics.

The proposed cDNA microarray will be spotted with purified RT-PCR products, from the 5'NCR and the VP4/VP2 regions of HRV prototype as well as HRV clinical isolates, on aminosilane coated glass slides. Microarrays will then be hybridized with fluorescently labeled test cDNA to detect and subtype human rhinoviruses based on unique hybridization patterns and signal strength. In the future, the cDNA microarray platform can be further expanded to include additional respiratory viruses, such as influenza and enteroviruses, to create a respiratory diagnostic panel.

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CHAPTER II

MOLECULAR DETECTION AND GENOTYPING OF HUMAN RHINOVIRUSES BASED ON THE 5' NON-CODING REGION

MOLECULAR DETECTION AND GENOTYPING OF HUMAN RHINOVIRUSES BASED ON THE 5' NON-CODING REGION

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Abstract

Human rhinoviruses (HRV), belonging to the *Picornaviridae* family, are the most frequent etiological agents of the common cold. Additionally, human rhinoviruses have been associated with more severe respiratory exacerbations in young children, elderly, and immunocompromised patients. HRVs are non-enveloped, icosahedral, single-stranded, positive-polarity RNA viruses with a genome of approximately 7.1 kb that includes a 5'noncoding region (5'NCR). The 5'NCR contains *cis* - elements that are involved in viral replication and translation of viral RNA, and is fairly conserved for picornaviruses. Since, there are greater than 100 distinct serotypes of HRVs, traditional serotyping to type HRVs is tedious, time-consuming, and inefficient. Molecular based genotyping assays provide an effective alternative to identify and differentiate between HRV strains. Molecular genotyping can be used to correlate specific strains to specific illnesses, provide meaningful epidemiological data, and help identify targets for future therapies.

We have developed a reverse transcription polymerase chain reaction (RT-PCR) based assay targeting the 5'NCR to genotype HRVs in clinical samples. Genomic sequences within 5' NCR were determined for all human rhinovirus prototype strains and used to assess their phylogenetic relationships. Sequence identity comparisons of clinical isolates with prototype strains were used to genotype clinical isolates. Using this approach, all clinical isolates associated with a single HRV prototype strain, with the exception of one isolate that may potentially be a novel HRV strain. Our results suggest

that the 5'NCR is a sensitive, specific target for rapid and cost effective means to genotype HRVs in clinical samples.

Introduction

Human rhinovirus (HRV), belonging to the *Picornaviridae* family, are responsible for causing a majority of the common colds with symptoms of rhinitis, fever, sore throat, and general malaise (8, 25). HRVs are non-enveloped, icosahedral, positive-polarity RNA viruses which have a single stranded genome of approximately 7.1 kb that includes a terminal VPg protein, a 5' non-coding region (5'NCR), genes encoding structural and non-structural proteins, followed by a 3' non-coding region (3'NCR), and a polyA tail (24). There are greater than 100 distinct serotypes of HRV that are divided into two groups based on host-cell receptor specificity. Major receptor group HRVs infect human respiratory epithelial cells using the intracellular adhesion molecule (ICAM) receptor (15, 30). Minor receptor group HRVs utilize low density lipoprotein receptors (LDLR) to infect host cells (12). Phylogenetic studies based on the analysis of VP4/VP2 region of all prototype strains further divide human rhinoviruses into two groups, HRV-A (consisting of 76 serotypes) and HRV-B (consisting of 25 serotypes) (27). Only HRV type 87 clusters individually and further characterization has revealed that it contains features of both HRV and the closely related human enterovirus (HEV) type 68, therefore, is classified as HEV-D (6).

Although most rhinovirus infections are self-limiting acute upper respiratory tract infections, recent studies suggest that HRVs can replicate in the lower respiratory tract and cause more severe disease in infants, elderly, and immunocompromised patients (2,

4, 9). HRVs have been associated with pneumonia, sinusitis, otitis media, and exacerbations of asthma and chronic obstructive pulmonary disease in high risk populations (3, 10, 11, 18, 22, 23). Early childhood rhinovirus infections have been linked to an increased risk of childhood asthma and allergic diseases (19). Recent HRV outbreaks in long term care facilities for the elderly persons have been associated with unusually high morbidity and high mortality (11, 20, 32).

Traditional laboratory diagnosis to identify HRV in clinical specimens is based on protection of cells *in vitro* by virus - neutralizing antibodies (14). Cell culture based assays are relatively costly, time-consuming, tedious, and impractical due to the great number of HRV serotypes and limited supply of immune sera (26). Alternatively, molecular based assays present cost effective, timely, and efficient tools for characterizing HRV (1, 5, 13, 21, 31). Previous molecular assays based on 3DPol, VP1, 2A, VP4/VP2 regions have been used to characterize HRV genus phylogeny (16, 17, 27, 28). Limited evaluation of the VP4/VP2 region has been performed for genotyping of HRV clinical isolates (29). However, the variability in conservation of these regions across the HRV genus can decrease specificity and the sensitivity of molecular diagnostics.

The 5'NCR was considered as a candidate region for reverse transcription polymerase chain reaction (RT-PCR) based genotyping of HRV in clinical samples. This region contains *cis* - elements involved in viral replication and translation of viral RNA and is fairly conserved within the *Picornaviridae* family (7). The 5'NCR contains an internal ribosomal entry site (IRES) that is used by the virus to translate its genome in a

cap-independent method. The IRES has distinct clover-leaf like structural features that can be used to distinguish HRVs from the closely related HEVs (33). As with a previous study within the VP4/VP2 region (27), genomic sequences in the 5'NCR can be used to assess phylogenetic relationships among the prototype strains. Consequently, sequence homology comparisons between the prototype strains and clinically isolated strains can be efficiently used to genotype clinical isolates (29). The purpose of this study is to use phylogenetic analyses of sequence comparisons within the 5'NCR to characterize and subtype HRV clinical isolates.

Materials and Methods

Virus strains. Seventy - six prototype HRV strains and 97 clinical HRV isolate strains were provided by the Viral and Rickettsial Disease Laboratory at the California Department of Health Services (Richmond, CA).

Media and growth conditions. To ensure good working titers, virus was expanded by infecting a confluent monolayer of human fetal diploid lung cells (maintained in 98% MEM Eagle with Hanks, 2% FCS) at 33°C until full cytopathic effects were observed (for up to 2 weeks). Cultures were frozen at -80°C and thawed at room temperature three times and centrifuged at 2000 Gs for 15 minutes at room temperature to release virus and remove cell debris. The cell culture supernatant containing virus (in same media) was aliquoted in 1 ml quantities and stored at -80°C.

Viral total RNA extraction. Total viral RNA was extracted from 150 μl of cell culture supernatant using QIAamp Viral RNA Mini Spin Kit (Qiagen, Valencia, CA) as per manufacturer instructions.

Reverse transcription polymerase chain reaction. First strand cDNA was synthesized using 5 μl of extracted viral RNA, random hexamer primers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), as per manufacturer instructions. Primers for PCR amplification of a fragment within the 5'NCR were designed based on an alignment of available HRV 5'NCR sequences within the GenBank database (NCBI). Forward primer RV179F 5'–CAAGCACTTCTGTTTCCC–3' and reverse primer RV568R 5'–CACGGACACCCAAAGTAGT–3', were used to PCR amplify a region within the 5'NCR using 2 μl of cDNA template 2.5 U of PfuTurbo DNA polymerase (Stratagene, Cedar Creek, TX), 0.8 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 1 μl of 100mM dNTP set (Invitrogen, Carlsbad, CA) in a final reaction volume of 50 μl. PCR conditions were as follows: hot start at 95°C (5 min) followed by forty cycles of denaturation at 95°C (15 s), annealing at 55°C (15 s), and elongation at 72°C (60 s) resulting in amplification of fragments approximately 350-400 nt in length.

Gel electrophoresis. Five microliters of each RT-PCR product was mixed with 1 μl of 6X loading buffer and subjected to 1% agarose gel electrophoresis. The gels were visualized after staining with ethidium bromide.

Purification and sequencing of PCR products. PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced in both directions using Sanger dideoxy cycle sequencing method with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) as per manufacturer instructions. Published HRV sequences within the 5'NCR were obtained from GenBank database (NCBI).

Sequence alignment and phylogenetic analyses. Sequences were aligned using ClustalX (v1.83). Multiple sequence alignment was subjected to phylogenetic analyses using programs in the PHYLIP package (v3.6). Distance matrices were calculated using DNAdist. Data was bootstrapped using SEQBOOT, and phylogenetic relationships were assessed using neighbor-joining, maximum parsimony, and maximum likelihood methods. Consensus trees were computed using CONSENSE and phylogenetic trees were visualized using TREEVIEW (v1.6.6).

Results

All HRV prototype strains had unique genomic sequences in the 5'NCR and clustered into two groups, HRV-A and HRV-B (Figure 2). Further, sequence identity comparisons between the prototype strains and clinically isolated strains led to association of clinical isolates with a single prototype strain of HRV, with the exception of one isolate, BMT-303 (Figure 3). In total, 82 clinical isolates were 5'NCR RT-PCR positive, whereas, previously, only 79 clinical isolates were VP4/VP2 RT-PCR positive. Comparison with the VP4/VP2 RT-PCR and the 5'NCR RT-PCR indicate that 5'NCR RT-PCR was able to detect a higher percentage of HRV (84.54%) than VP4/VP2 RT-PCR (72.1%) in clinical specimens. There was a high correlation (98.57%) of 5'NCR results with previous VP4/VP2 results. A single isolate, V06T01482, differed with respect to VP4/VP2 and 5'NCR RT-PCR results. Also, in certain cases, we found that the 5'NCR RT-PCR could effectively detect and differentiate between HRV and HEV.

Discussion

Our results indicate that RT-PCR targeting the 5'NCR can be efficiently used to infer phylogenetic relationships between HRV prototype strains. Based on the 5'NCR, all HRV prototype strains clustered into two groups, HRV-A and HRV-B, as previously reported with VP4/VP2 analysis (27). Sequence homology between HRV prototype strains and HRV clinical isolates can be effectively used to genotype HRV clinical isolates. Except for a single isolate, BMT-303, all clinical isolates clustered with a single prototype strain. BMT-303, isolated from a bone marrow transplant recipient, may represent a novel strain of human rhinovirus based on sequence divergence. We are in the process of further characterizing this isolate.

The high correlation between previously evaluated VP4/VP2 RT-PCR and 5'NCR RT-PCR implies that sequences within the 5'NCR are strain specific and can be used to subtype HRVs. A single discrepancy, V06T01482, may have occurred because of multiple HRV strains in the clinical sample as result of a coinfection. Additionally, we found that 5'NCR RT-PCR was more sensitive and specific than the VP4/VP2 RT-PCR, allowing detection and genotyping of 12 additional isolate strains. In a few cases, the 5'NCR could effectively differentiate between HRV and the closely related genera, HEV.

Although, additional studies correlating molecular genotyping results with traditional neutralization assays are needed, a molecular approach for genotyping HRV clinical isolates can facilitate collection and analysis of epidemiological data. Future studies that can reliably genotype clinical isolates may allow correlation of specific HRV

strains with more severe symptoms, as well as, allow for the discovery and characterization of novel strains.

Acknowledgments

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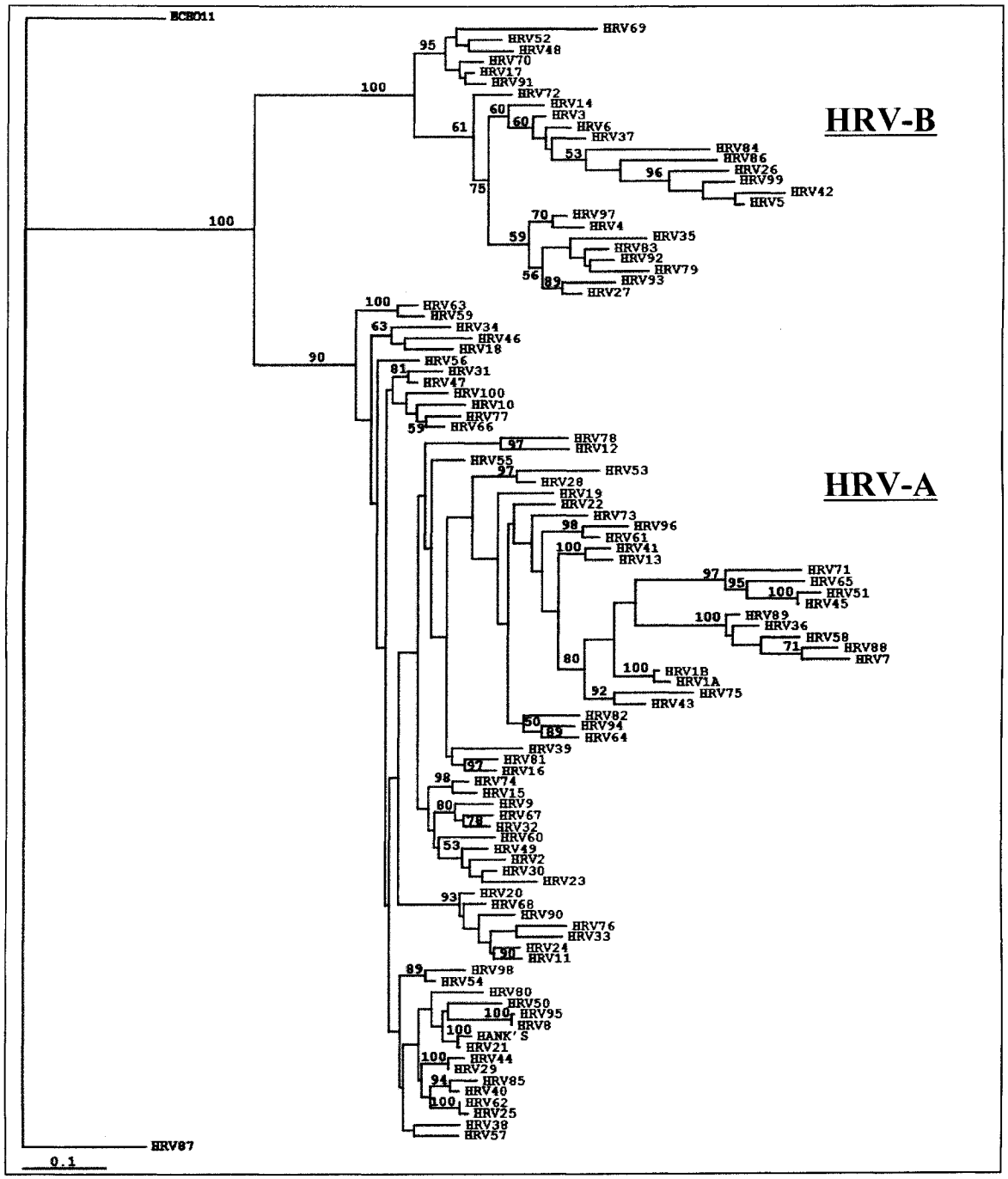

Figure 2. Phylogenetic clustering of HRV prototype strains based on the 5'NCR. Prototype strains cluster into Group A and B as expected.

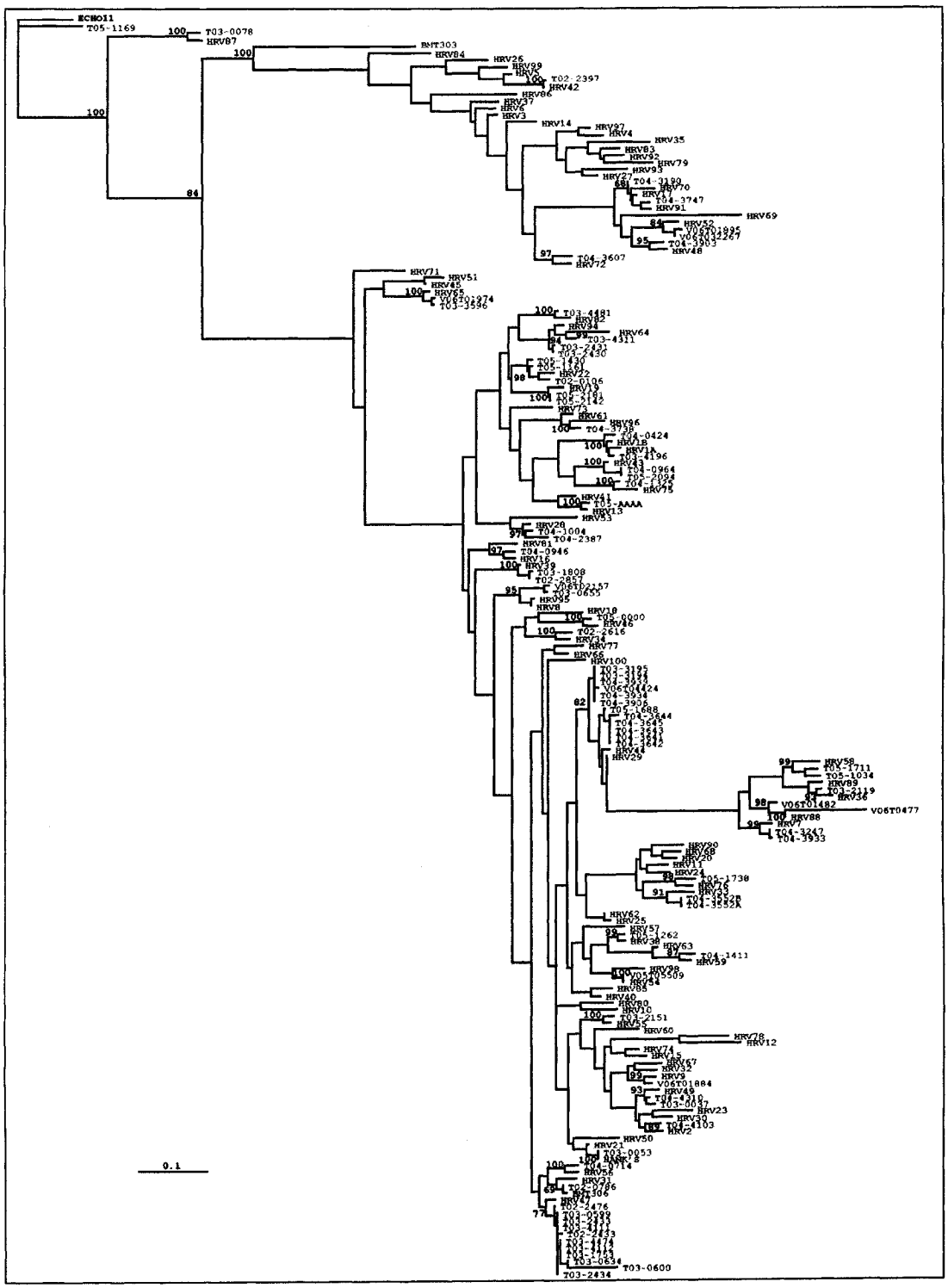

Figure 3. Phylogenetic clustering of clinical isolates with HRV prototype strains based on 5'NCR sequence homology. Prototype strains are highlighted in purple and clinical isolates in red.

CHAPTER III

USE OF MICROARRAY TECHNOLOGY FOR SIMULTANEOUS DETECTION AND GENOTYPING OF HUMAN RHINOVIRUSES

USE OF MICROARRAY TECHNOLOGY FOR SIMULTANEOUS DETECTION AND GENOTYPING OF HUMAN RHINOVIRUSES

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Abstract

Use of microarray technology to simultaneously detect and genotype human rhinoviruses (HRVs) based on the 5' non-coding region (5'NCR) and VP4/VP2 region was evaluated. Microarrays, containing RT-PCR products from 5'NCR and VP4/VP2 regions of various prototypes and clinically isolated HRV strains, were constructed. Each sample was represented in triplicate on two subarrays and tested against labeled cDNA from known prototype strains and previously genotyped clinical isolates. Unique and selective hybridization patterns were seen for each tested strain. Preliminary results indicate that microarrays have the potential to provide a highly efficient and high throughput platform for simultaneous detection and genotyping of HRVs.

Introduction

Microarray technology uses precision robotics to produce microscopic arrays of immobilized nucleic acids on glass slides, silicone chips, or nylon membranes (2). Such technology takes advantage of sequence specific hybridization of nucleic acids and is applicable to fields of genomics, drug discovery, vaccine design, and evolution (1, 3, 6, 10). Microarrays have revolutionized viral diagnostics by allowing rapid testing of clinical specimens to identify and subtype viral pathogens (4, 5, 7-9). This approach can provide a highly efficient, sensitive and specific platform for simultaneous screening of multiple pathogens in the routine clinical setting.

Due to the great number of serotypes of HRVs, traditional laboratory diagnostics to characterize HRVs in clinical samples can be tedious, time-consuming, and inefficient. Microarrays offer an attractive alternative to effectively detect and type HRVs because

such technology can greatly reduce workload in a laboratory setting. Possibility of high capacity and high throughput analysis can also lead to collection of meaningful epidemiological data correlating specific HRV strains with more severe illnesses or epidemics. Microarrays can also be used to detect novel strains of virus based on hybridization of conserved sequences. This study describes the development of a cDNA microarray for detecting and genotyping HRVs based on the 5'NCR and VP4/VP2 target regions.

Materials and Methods

Virus strains. Seventeen prototype HRV strains and 15 clinical HRV isolate strains were provided by the Viral and Rickettsial Disease Laboratory at the California Department of Health Services (Richmond, CA).

Cloning RT-PCR products into plasmid vector. Previously purified RT-PCR products from the 5'NCR and VP4/VP2 regions were inserted into commercial pCR4TOPO plasmid vector (Invitrogen, Carlsbad, CA) by T/A cloning and introduced into TOP10 *E.coli* (Invitrogen, Carlsbad, CA), as per manufacturer instructions. Bacterial cells were plated on Luria broth agar containing ampicillin and incubated overnight at 37°C. Colonies were subsequently screened by PCR using primers T3 (5′-ATTAACCCTCACTAAAGGGA-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′).

Gel electrophoresis. Five microliters of each PCR product was mixed with 1 μl of 6X loading buffer and subjected to 1% agarose gel electrophoresis. The gels were visualized after staining with ethidium bromide.

Purification and sequencing of PCR products. Positive PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). To ensure sequence of insert was correct, PCR products were sequenced in both directions using Sanger dideoxy cycle sequencing method with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) as per manufacturer instructions.

Microarray spotting. Aminosilane coated glass slides (Erie Scientific Company, Portsmouth, NH) were spotted with purified PCR products using 100 μm ChipMaker Micro Spotting Device (TeleChem International, Inc., Sunnyvale, CA) and microarrayer (courtesy of Relman Lab, Stanford University, Palo Alto, CA). Each sample was spotted in triplicate on two subarrays per slide. Plasmid vector pUC19 was used as a negative control.

Synthesis and purification of Cy-3 labeled test cDNA. For each labeling reaction, 5 μl of extracted viral RNA was used in the presence of 1 μg/μl random hexamer primer, 1 mM Cy3-dUTP (Amersham Biosciences, Pittsburgh, PA), 25 mM dNTPs, 0.1 M DTT, First Strand Buffer (Invitrogen, Carlsbad, CA) and 400 U SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and incubated for one hour. After one hour incubation, 200 U of SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) was added and the reactions were incubated for another hour. Reactions were stopped by adding 0.1 N NaOH and neutralized by adding 0.1 N HCl. Human metapneumovirus was used as a negative control. Labeled cDNA was purified

using MinElute Reaction Cleanup Kit (Qiagen, Valencia, CA) as per manufacturer instructions.

Hybridization of Cy-3 labeled test cDNA to the microarray. The test cDNA was applied to the microarray and incubated in a hybridization cassette (TeleChem International, Inc., Sunnyvale, CA) overnight at 65°C. After incubation arrays were removed and washed at room temperature in 2X SSC, 0.03% SDS for approximately 2 min. Microarrays were further washed in 2X SSC, 1X SSC, and 0.2X SSC for 2 min. Subsequently, slides were dried by centrifugation at 25 Gs for 2 min.

Scanning and analysis of microarrays. Dried arrays were scanned at 532 nm and images were acquired on a GenePix 4000B Microarray Scanner (Axon Instruments, Union City, CA). Images were aligned and analyzed with GenePix Pro software (Axon Instruments, Union City, CA) based on signal strength.

Results

Low background and autofluorescence was observed for each tested microarray. No signal was received for microarrays hybridized with the negative control cDNA from human metapneumovirus (data not shown). Unique and selective hybridization pattern was seen for each tested HRV strain (Figure 4) allowing correct identification and subtyping of test cDNA. Stronger signals were seen for spots containing VP4/VP2 RT-PCR products, although results for both 5'NCR and VP4/VP2 regions were in agreement and gave strong signals.

Discussion

High hybridization signals combined with low background indicate that the microarray is highly specific for detecting HRV cDNA. Each test cDNA was correctly subtyped and was in agreement with previous 5'NCR RT-PCR and VP4/VP2 RT-PCR results. There was a strong agreement between VP4/VP2 and 5'NCR results, as expected. The stronger hybridization of test cDNA with VP4/VP2 products on the microarray may be due to the larger fragment size of the VP4/VP2 PCR products. The VP4/VP2 PCR products are approximately 540 nt long, whereas the 5'NCR PCR products are approximately 350 nt long. HRV isolate T03-2119 test cDNA hybridized strongly with spots containing 5'NCR and VP4/VP2 from HRV 36, hence genotyping T03-2119 as HRV36. These results are in agreement with previous RT-PCR based genotyping results.

Our results support the potential use of microarrays for detecting and genotyping HRV in clinical samples. This is the first report using cDNA microarray technology for effective characterization of HRVs. Further evaluation of microarray are required, but these preliminary results indicate that cDNA microarrays can be a highly efficient and high throughput platform for simultaneous detection and genotyping of HRVs.

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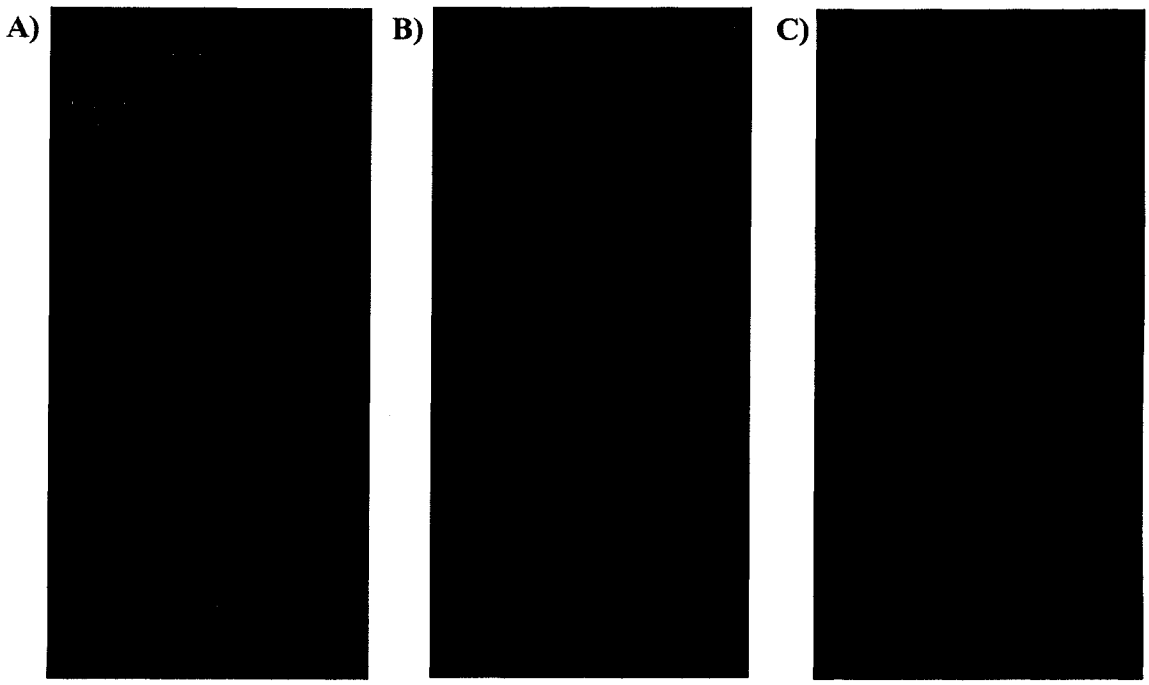

Figure 4. Unique and selective hybridization patterns seen for each test cDNA. A) test cDNA from HRV 4, member of HRV-B. B) test cDNA from HRV 71, member of HRV-A. c) test cDNA from clinical isolate T03-2119, previously genotyped as HRV 36, was correctly subtyped as HRV 36 based on microarray analysis.

APPENDIXES

APPENDIX A. ADDITIONAL REFERENCES

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APPENDIX B. WORKFLOW DIAGRAM FOR RT-PCR

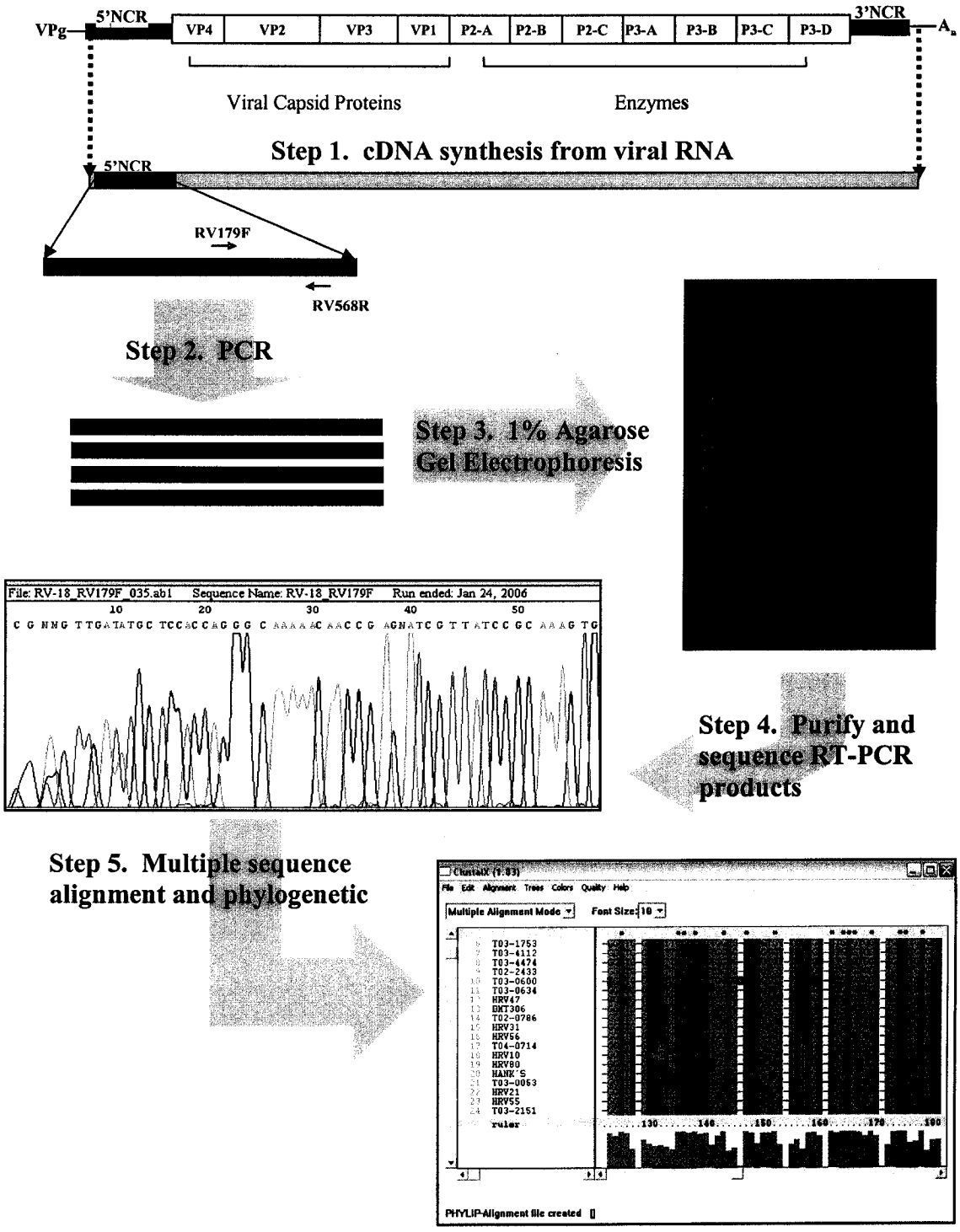

Figure 5. Workflow schematic of 5'NCR RT-PCR for detection and genotyping of HRV

APPENDIX C. WORKFLOW DIAGRAM FOR MICROARRAY

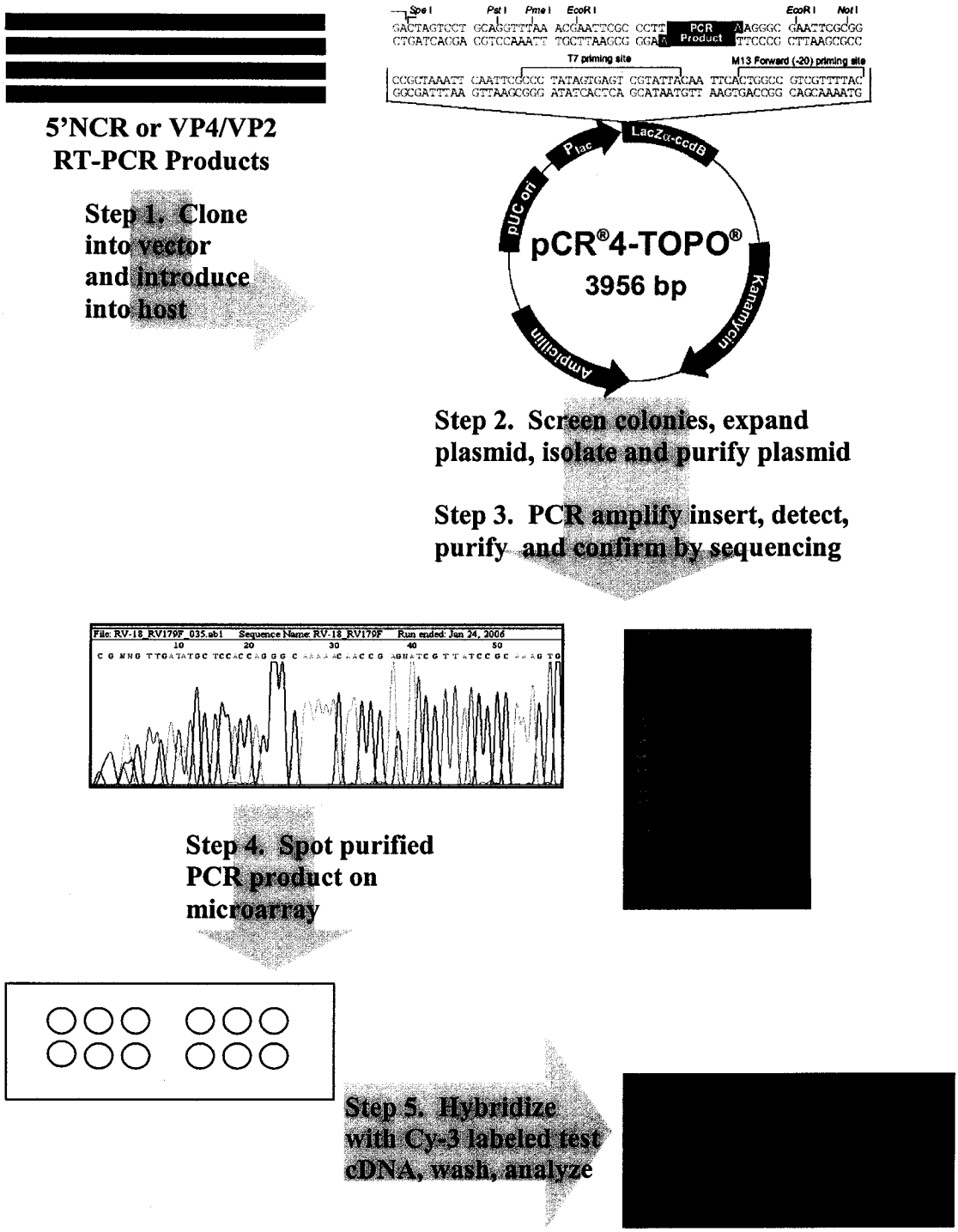

Figure 6. Workflow schematic of microarray platform for characterizing HRV

APPENDIX D. SUMMARY OF RT-PCR RESULTS

Table 1. 5'NCR RT-PCR results correlating with VP4/VP2 RT-PCR results.

HRV Isolate ID	HRV Type	HRV Isolate ID	HRV Type
BMT306	HRV31	T04-2387	HRV28
T02-0106	HRV22	T04-2896	RT-PCR negative
T02-0786	HRV31	T04-3190	HRV70
T02-0928	RT-PCR negative	T04-3247	HRV7
T02-0968	RT-PCR negative	T04-3462	RT-PCR negative
T02-1301	RT-PCR negative	T04-3552A	HRV33
T02-2397	HRV42	T04-3552B	HRV33
T02-2433	HRV47	T04-3607	HRV72
T02-2476	HRV47	T04-3641	HRV29
T02-2477	RT-PCR negative	T04-3642	HRV29
T02-2616	HRV34	T04-3643	HRV29
T02-2857	HRV39	T04-3644	HRV29
T03-0037	HRV49	T04-3645	HRV29
T03-0053	HRV Hanks	T04-3738	HRV61
T03-0066	RT-PCR negative	T04-3747	HRV91
T03-0078	HRV87	T04-3900	RT-PCR negative
T03-0599	HRV47	T04-3901	RT-PCR negative
T03-0600	HRV47	T04-3903	HRV48
T03-0634	HRV47	T04-3906	HRV44
T03-0655	HRV95	T04-3933	HRV7
T03-1753	HRV47	T04-3934	HRV44
T03-1808	HRV39	T04-3935	RT-PCR negative
T03-2119	HRV36	T04-3936	RT-PCR negative
T03-2151	HRV55	T04-3937	RT-PCR negative
T03-2430	HRV94	T04-3938	RT-PCR negative
T03-2431	HRV94	T04-3939	HRV44
T03-2434	HRV47	T04-4103	HRV2
T03-3194	HRV44	T04-4113	RT-PCR negative
T03-3596	HRV65	T04-4310	HRV49
T03-4111	HRV47	T05-0000	HRV46
T03-4112	HRV47	T05-1262	HRV38
T03-4196	HRV1B	T05-1430	HRV22
T03-4311	HRV94	T05-1688	HRV29
T03-4474	HRV47	T05-1738	HRV76
T03-4481	HRV82	T05-1746	RT-PCR negative
T04-0424	HRV1B	T05-2142	HRV19
T04-0714	HRV56	T05-2181	HRV19
T04-0946	HRV16	T05-AAAA	HRV13
T04-0964	HRV43	V06T01884	HRV9
T04-1004	HRV28	V06T01895	HRV52

Table 1 (cont'd). 5'NCR RT-PCR results correlating with VP4/VP2 RT-PCR results.

HRV Isolate ID	HRV Type	HRV Isolate ID	HRV Type
T04-1325	HRV75	V06T01974	HRV65
T04-1411	HRV59	V06T02157	HRV8

Table 2. 5'NCR RT-PCR results that do not correlate with VP4/VP2 RT-PCR results.

RV Isolate	5'NCR RT-PCR	VP2/VP4 RT-PCR
V06T01482	HRV88	HRV63

Table 3. 5'NCR RT-PCR positive isolates that were VP4/VP2 RT-PCR negative.

RV Isolate	5'NCR RT-PCR	VP2/VP4 RT-PCR
BMT303	Positive, untypable	RT-PCR negative
T03-2433	HRV47	RT-PCR negative
T03-3195	HRV44	RT-PCR negative
T05-1034	HRV58	RT-PCR negative
T05-1161	HRV22	RT-PCR negative
T05-1169	Human enterovirus 71	RT-PCR negative
T05-1711	HRV58	RT-PCR negative
T05-2094	HRV43	RT-PCR negative
V06T03226,7	HRV52	RT-PCR negative
V06T04424	HRV44	RT-PCR negative
V06T0477	HRV88	RT-PCR negative
V06T05509	HRV54	RT-PCR negative

APPENDIX E. EXPANDED LABORATORY PROTOCOLS

First strand cDNA synthesis

I. Add 1 µl of 50-250 ng random hexamer primer, 1 ng - 5 µg total RNA, 1 µl dNTP Mix (10 mM each), and sterile, distilled water to 12 µl.
II. Heat mixture to 65°C for 5 min and quick chill on ice. Collect contents of tube by brief centrifugation and add 4 µl of 5X First-strand Buffer, 2 µl of 0.1M DTT and incubate at 42°C for 2 minutes.
III. Add 1 µl (200 U) of SuperScript II RT and mix by pipetting gently up and down.
IV. Incubate at 42°C for an hour.
V. The cDNA can be used as template for amplification in PCR.

Polymerase chain reaction

I. Add following to PCR tube:

10X Buffer	5 µl
Forward Primer	1 µl
Reverse Primer	1 µl
10 mM dNTPs	1 µl
Ultra pure water	40 µl
Taq polymerase	0.2 µl
PFU polymerase	0.5 µl
cDNA template	2 µl

II. Mix gently and heat reaction to 95°C for 2 min to denature.
III. Perform 40 cycles of PCR as follows
   a. 95°C – 15 s
   b. 50°C – 15 s
   c. 72°C – 1 min

Gel electrophoresis

I. Prepare 1 % agarose gel and set up electrophoresis unit.
II. Prepare samples (5 µl product with 1 µl of 6X loading buffer).
III. Load samples (~6 µl per lane). Load 10 µl KB+ ladder into reference lanes.
IV. Run gel at 80V for ~1 hour using 1X TAE buffer.
V. After run, stain gel with EtBr for 5 min, destain with DI H_2O for 10-15 minutes.
VI. Capture image. Purify any amplification product for sequencing.

Cloning purified RT-PCR products into electrocompetent TOP10 *E. coli*

I. Mix following reagents in sterile microfuge tube

Purified RT-PCR Product	4 µl
Dilute salt solution	1 µl
H_20	2 µl
pCR4TOPO vector plasmid	1 µl

II. Mix gently and incubate for 5 minutes at room temperature.
III. Place reaction on ice until ready for transformation.

Transformation of electrocompetent TOP10 *E. coli*

I. Mix together in labeled sterile 2.5 ml capped tube. Mix gently by tapping side of tube. Keep on ice.

Cloned product	2
TOP10 *E. coli*	25
H_20	13

II. Add mixture to 0.1 cm cuvette. Have 900 µl media (S.O.C or LB) ready.
III. Electroporate by pulsing at 1.8 kV, 25µF, and 200 ohms.
IV. Immediately add media, mix, and transfer all the cells to culture tube.
V. Incubate in shaker at 37°C for about one hour.
VI. Plate 10-50 µl of each sample onto pre-warmed selective plates. Incubate overnight.
VII. Select about 10 colonies for screening by PCR by mixing with sterile saline.

Screening colonies for cloned vector by PCR

I. Add following to PCR tube:

10X Buffer	5 µl
Forward Primer	1 µl
Reverse Primer	1 µl
10 mM dNTPs	1 µl
Ultra pure water	40 µl
Taq polymerase	0.2 µl
PFU polymerase	0.5 µl
Cells from colony	1 µl

II. Mix gently and heat reaction to 95°C for 2 min to denature.
III. Perform 40 cycles of PCR as described above.

Expanding positive clones for plasmid prep

I. Add ~10 ml of selective media into sterile culture tube.
II. Add 50 µl of culture from positive saline
III. Incubate overnight in shaker at 37°C.
IV. Aliquot 0.8 ml of culture and freeze in 0.2 ml 75% glycerol as stock.
V. Rest of the culture can be centrifuged (8000 Gs for 10 minutes) and used for plasmid prep as per manufacturer's instructions.

Preparing fluorescently labeled cDNA probe

I. To anneal primers, mix following in thermocycler tube

Total RNA	50 - 100 µg
Random Hexamer	4 µg
ddH_20	to 15.4 µl

II. Heat mixture to 65°C for 10 minutes and cool on ice
III. Add 14.6 µl of following mixture to each reaction tube

5X First Strand Buffer	6 µl
0.1M DTT	3 µl
Cy3 (1mM)	3 µl
Unlabeled dNTPs*	0.6 µl
Superscript II (200 U/µl)	2 µl

IV. Incubate 42°C for 1 hour.
V. Add 1 µl of SSII (RT booster). Incubate 42°C for 1 hour.
VI. Degrade RNA and stop reaction by adding 15 µl of 0.1N NaOH.
VII. Incubate at 70°C for 30 min.
VIII. Neutralize by adding 15 µl of 0.1N HCl

* Unlabeled dNTPs:
dATP (25 mM)
dCTP (25 mM)
dGTP (25 mM)
dTTP (10 mM)

Probe clean-up

I. Add 230 µl of Qiagen Buffer PBI to fluorescent labeled cDNA. Mix well by pipetting.
II. Add mixed contents to a Qiagen MinElute column and spin for 1 minute at 10,000 g. Discard flow-through.
III. Add 750 µl of Qiagen Buffer PE to column and spin for 1 minute at 10,000 g. Discard flow-through
IV. Spin for 1 minute at maximum speed to remove residual wash buffer
V. Place column in a clean tube and add 12 µl of H_2O to the center of filter. Incubate 2 minutes at room temperature
VI. Spin for 1 minute at maximum speed to elute

Prehybridization

I. UV cross-link printed DNA onto glass substrate with 65 mJ of energy in Stratalinker (650 x 100µJ) and transfer arrays on a slide rack.
II. Prepare prehybridization buffer (5X SSC, 0.1 mg/ml BSA, 0.1% SDS)

ddH_20	300 ml
20X SSC	100 ml
10 mg/ml BSA	4 ml
10% SDS	4 ml

III. Flood slide with prewarmed (42°C) prehybridization buffer (5X SSC, 0.1mg/ml BSA, 0.1% SDS) for 1 hour
IV. Rinse arrays in 0.1X SSC for 5 minutes at room temperature
V. Transfer arrays to MilliQ water and incubate at room temperature for 30 seconds
VI. Wash once more in 0.1X SSC for 5 minutes at room temperature
VII. Transfer arrays to MilliQ water and incubate at room temperature for 30 seconds
VIII. Dry arrays and use same day

Hybridization of Cy-3 labeled test cDNA with microarray

I. Measure probe volume with pipettor and add water to bring volume to 14.8 µl.
II. Add 2.7 µl 20X SSC. Mix well.
III. Add 0.54 µl of 10% SDS and mix well. Centrifuge briefly to collect entire volume to the bottom of the tube.
IV. Prepare hybridization chamber by adding 20 µl of 3X SSC to each reservoir.
V. Place 18 µl of prepared probe off center on the array and cover gently with coverslip. Place slide into hybridization chamber.
VI. Tightly secure hybridization chamber and place in 65°C water bath overnight.

Washing arrays after hybridization

I. Prepare Wash Solutions:

Solution #1: 200 ml (2X SSC, 0.03% SDS)
Solution #1B: 200 ml (2X SSC)
Solution #2: 200 ml (1X SSC)
Solution #3: 200 ml (0.2X SSC)

II. Remove hybridization chamber from water bath and extract slide and immediately submerge into Solution #1, tilting and allowing the coverslip to slide off. Wash slides by agitation for 2 min.
III. Remove each slide and wash in Solution #2 for 2 min.
IV. Remove each slide and wash in Solution #3 for 2 min.
V. Spin dry by centrifugation in a slide rack at 25 Gs for 2 min.
VI. Scan arrays at 532 nm (Cy3) immediately.

APPENDIX F. LIST OF 5'NON-CODING REGION SEQUENCES

>HRV3 5'NCR
TATCCGCCAACCAACTACGTAAAAGCTAGTAGTATTATGTTTTTAACTAGGCG
TTCGATCAGGTGGATTTCCCCTCCACTAGTTTGGTCGATGAGGCTAGGAATTC
CCCACGGGTGACCGTGTCCTAGCCTGCGTGGCGGCCAACCCAGCTTATGCTG
GGACGCCTTTTTATAGACATGGTGTGAAGACTCGCATGTGCTTGGTTGTGATT
CCTCCGGCCCCTGAATGCGGCTAACCTTAACCCTGGAGCCTTGTGTCACAAAC
CAGTGATGATAAGGTCGTAATGAGCAATTCCGGGACGGGACCGACTA

>HRV4 5'NCR
TATCCGCCAATCAACTACGTAACGGCTAGTATCATCTTGCTTTTGATTTGGTG
TTCGATCAGGTGGTATCCCCCACTAGTCTGGTCGATGAGGCTAGGAATTCCCC
ACGGGCGACCGTGTCCTAGCCTGCGTGGCGGCCAGCCCAGCTTTTGCTGGGA
CGCCTTTTCAAAGACATGGTGTGAAGACCTGCATGTGCTTGGTTGTGAGTCCT
CCGGCCCCTGAATGCGGCTAACCTTAACCCTGGAGCCCAGCAGCATAATCCA
ATGTTGTTTGGGTCGTAATGAGCAATTCCGGGACGGGACGCAGT

>HRV5 5'NCR
TATCCGCCAGCTAACTGCGAGAAGACTAGTAACACCATGTTTGTTTTTAGACG
TTCGATCAGGTGAATACCCCATTCACTAGTTTGGTCGATGAGGCTGAGAATAC
CCCACAGGTAACTGTGTCTCAGCCTGCGTGGCGGCCAACCCGACTCATGTCG
GGACGCCTACTAATAGACATGGTGTGAAGATCCTATTGCGCTTAGTTGTGAGT
CCTCCGGCCCCTGAATGCGGCTAACCCTAACCCCGGAGCCTTGTGGTGTAAA
CCAACATTTGCAAGGTCGTAATGAGTAATTCTGGGATGGGACCGACTA

>HRV8 5'NCR
TATCCGCAAGATGCCTACGCAAAGCTTAGTAACACTTTGGAAGATTTATGGCT
GGTCGTTCCACTATACCCCATAGTAGACCTGGCAGATGAGGCTAGAAATACC
CCACTGGTGACAGTGTTCTAGCCTGCGTGGCTGCCTGCGCACCCTATGGGTGC
GAAGCCATACATTGGACAAGGTGTGAAGAGCCCCGTGTGCTCATCTTGAGTC
CTCCGGCCCCTGAATGTGGCTAACCTTAACCCCGCAGCCAGTGCACACAATC
CAGTGTGTTTTTGGTCGTAATGAGTAATTGTGGGATGGGACCAACT

>HRV9 5'NCR
TATCCGCAAAGCGCCTACGCAAAGCCTAGTAACTATCTGGAAGTTGCTTGGTT
GGTCGCTCCGCCATATCCCATGGTAGACCTGGCAGATGAGGCTAGAAATTCC
CCACTGGTGACAGTGTTCTAGCCTGCGTGGCTGCCTGCACACCCTCTGGGTGT
GAAACCAAGTAATGGACAGGGTGTGAAGAGCCCCGTGTGCTCGCTTTGAGTC
CTCCGGCCCCTGAATGTGGCTAACCTTAACCCTGCAGCTAGGGCACACAATC
CAGTGTGTATCTAGTCGTAATGAGCAATTGCGGGATGGGACATTGG

>HRV10 5'NCR
TACCCGCAAGGTGCCTACGCAAAGCCTAGTAACCCCTTGAACGATGTGTGGT
TGGTCGTTCGGCTGTACCCCACAGTAGACCTGGCAGATGAGGCTAGATGTTC
CCCACTGGCGACAGTGTTCTAGCCTGCGTGGCTGCCTGCGCACTCTCTGAGTG
CGAAGCCATACATTTGACAAGGTGTGAAGAGCCCCGTGTGCTCACCTTGAGT
CCTCCGGCCCCTGAATGTGGCTAACCTTAACCCTGCAGCTAGTACATGCAATC
CAGCATGCAGCTAGTCGTAACGAGCAATTGCGGGACGGGACAA

>HRV11 5'NCR
TATCCGCAAAGCAACTACGCAAAGCCTAGTATCACTTTTGAGAGTATGTGGTT
GGTCGCTCCACTATAAACCCAATAGTAGACCTGGCAGATGAGGCTAGAAGTC
CCCCACTGGTAACAGTGTTCTAGCCTGCGTGGCTGCCTGCGTGCCTCTGGTAG
GCACGAAGCCATATATTTGACAAGGTGTGAAGAGCCCCGTGTGCTTGCTTTG
AGTCCTCCGGCCCCTGAATGTGGCTAACCTTAACCCTGCAGCTAGTGTGCATA
ATCCAATGCATGGCTAGTCGTAATGAGCAATTGCGGGATGGGACCAAC

>HRV12 5'NCR
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GTGAAGNCAAATTTACTGACAGGGTGTGAAGAGCCTATTGTGCTCACCTTGA
TTCCTCCGGCCCCTGAATGTGGCTAATCCTAACCCCGCTGCTATTGCACACGA
ACCAGTGTGTAGATAGTCGTAATGAGCAATAGTGGGATGGAACCAACTAT

>HRV13 5'NCR
TACCCGCAAGGTGCCTACACAGAACTTAGTACCATTCTGGAAAATTCTTGGCT
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CCCCACTGGCGACAGTGTTCTAGCCTGCGTGGCTGCCTGCACACCCTTCTTGG
GTGTGAAGCCTAGAATTGGACAGGGTGTGAAGAGTCGCGTGTGCTCATCTTG
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>HRV15 5'NCR
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CCACTGGCGACAGTGTTCTAGCCTGCGTGGCTGCCTGCACACCCTTTGGGTGT
GAAGCCAAATTACGGACAAGGTGTGAAGAGCCCCGTGTGCTCACTTTGAGTC
CTCCGGCCCCTGAATGTGGCTAACCTTAACCCTGCAGCTAGAGTGCACAATC
CAGTGTATTTCTAGTCGTAATGAGCAATTGCGGGATGGGACA

>HRV18 5'NCR
TATCCGCAAAGTGCCTACGCAAAGCCTAGTAGCACTTTGAATAGCATGTGGT
TGGTCGCTCCACTATTCCCCATAGTAGACCTGGCAGATGAGGCTAGAAATCC
CCCACTGGCGACAGTGTTCTAGCCTGCGTGGCTGCCTGCGTGTCCTAACGGAC
ACGAAGCCATATGTTTGACAAGGTGTGAAGAGCCCCGTGTGCTCACTTTGAG
TCCTCCGGCCCCCGAATGTGGCTAACCTTAACCCTGCAGCTGGGGCATACAA
TCCAGTGTGAACTCAGTCGTAATGAGCAATTGCGGGATGGGACCA

>HRV19 5'NCR
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GGTCGCTCAAGTACAAACCCAGTACTAGACCTGGCAGATGAGGCTAGAAAAC
CCCCACTGGCGACAGTGTTCTAGCCTGCGTGGCTGCCTGCACACCCATTTGGG
TGTGAAGCCAAACAACGGACAGGGTGTGAAGAGCCACGTGTGCTCATCTTGA
TTCCTCCGGCCCCTGAATGCGGCTAACCTTAAACCTGCAGCCATTGCTCACAA
TCCAGTGAGTTGGTGGTCGTAATGAGCAATTGCGGGATGGGACCGACA

>HRV20 5'NCR
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GTTGGTCGTTCCACTATGAACCCGATAGTAGACCTGGCAGATGAGGCTAGAA
ATTCCCCACTGGCAACAGTGTTCTAGCCTGCGTGGCTGCCTGCGTACCCCCTG
TGGGTACGAAGCCATACATTTGACAAGGTGTGAAGAGCCCCGTGTGCTTGCT
TTGAGTCCTCCGGCCCCTGAATGTGGCTAACCTTAACCCTGCAGCTAGTGTGT
ACAATCCAGTACATAGCTAGTCGTAATGAGCAATTGCGGGATGGGACCA

>HRV22 5'NCR
TACCCGCAAGATGCCTACGCAAAGCTTAGTAACATCTTCGAAGATACTTAGTT
GGTCGCTCATGTGCAACCCCAGCACTAGACCTGGCAGATGAGGCTAGAAATT
CCCCACTGGCGACAGTGTTCTAGCCTGCGTGGCTGCCTGCACACCCTAACGG
GTGTGAAGCCCAGTATTGGACAAGGTGTGAAGAGCCCCGTGTGCTCATCTTG
AGTCCTCCGGCCCCTGAATGCGGCTAACCTTAAACCTGCAGCCATTGCTCACA
ACCCAGTGAGTCTATGGTCGTAATGAGCAATTGCGGATGACCG

>HRV23 5'NCR
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GGTCGTTCCACCATTCCCCCTGGTAGACCTGGCAGATGAGGCTAGAAACTCC
CCACTGGCGACAGTGTTCTAGCCTGCGTGGCTGCCTGCACACCCTCTGGGTGT
GAAGCCATACGATGGACAGGGTGTGAAGAGCCCCGTGTGCTCGCTTTGAGTC
CTCCGGCCCCTGAATGTGGCTAACCTTAACCCTGCAGCTAGAGCACACAATC
CAGTGTGTTTCTAGTCGTAATGAGCAATTGCGGGACGGGACAAGGT

>HRV24 5'NCR
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TTCCCCACTGGTAACAGTGTTCTAGCCTGCGTGGCTGCCTGCGTGCTCCTTGT
GAGCACGAAGCCATATATTTGACAAGGTGTGAAGAGCCCCGTGTGCTTGCTT
TGAGTCCTCCGGCCCCTGAATGTGGCTAACCTTAACCCTGCAGCTAGCGTGTA
CAATCCCAGTACAAAGCTAGTCCGTANTGAGCAATTGCGGGATGAC

>HRV25 5'NCR
TAACCGCAAAGTGACTACGCAAAGCTTAGTAATGCCTTGAAGAATTTATGAC
TGGTCGTTCCGCTATAACCCCTAGTAGACCTGGCAGATGAGGCTAGAAATAC
CCCACTGGTAACAGTGTTCTAGCCTGCGTGGCTGCCTGCACACCCTTTCGGGT
GTGAAGCCATATATTTGACAAGGTGTGAAGAGCCCCGTGTGCTCACTTTGAG
TCCTCCGGCCCCTGAATGTGGCTAACCTTAACCCTGCAGCTAGTGTATGTAAT
CCAACATATGGCTAGTCGTAATGAGCAATTGCGGGATG

>HRV26 5'NCR
TATCCTGCCAACCAACTACGAGGACGGCTAGGTAATATCATGTTTGTCTTTGA
GCGTTCGATCAGGTGAATTCCCCATTCACTAGTTTGGTCGATGAGGCTGAGAA
CTCCCCACAGGTGACTGTGTCTCAGCCTGCGTGGCGGCCAACCCAGCCACGG
CTGGGACGCCCACTGATAGACATGGTGTGAAGACCCAATTGTGCTTGGTTGT
GACTCCTCCGGCCCCTGAATGCGGCTAACCTCAACCCCGGAGCCTTGTAGTGT
AAGCCAACACATACAAGGTCGTAATGGGCAACTCTGGGACGGGACCGA

>HRV27 5'NCR
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TTCGATCAGGTGGTTCCCCCCACTAGTTTGGTCGATGAGGCTAGGAACTCCCC
ACGGGTGACCGTGTCCTAGCCTGCGTGGCGGCCAACCCAGCTTTTGCTGGGA
CGCCTTTTTACAGACATGGTGTGAAGACCTGCATGTGCTTGATTGTGAGTCCT
CCGGCCCCTGAATGCGGCTAACCTTAACCCCGGAGCCTTGCAACATAATACC
AATGTTGTTGAGGTCGTAATGAGTAATTCCTGGATGACCG

>HRV28 5'NCR
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>HRV31 5'NCR
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>HRV96 5'NCR
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>HRV97 5'NCR
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>HRV98 5'NCR
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>HRV99 5'NCR
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>HRV100 5'NCR
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>HANK'S 5'NCR
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>T04-3607 5'NCR
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>T04-3738 5'NCR
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>T04-3747 5'NCR
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>T04-3933 5'NCR
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>T04-4310 5'NCR
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>BMT303 5'NCR
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>BMT306 5'NCR
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>T05-2094 5'NCR
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>T02-0106 5'NCR
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>T02-0786 5'NCR
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>T02-2397 5'NCR
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>T02-2433 5'NCR
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>T02-2476 5'NCR
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>T02-2616 5'NCR
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>T02-2857 5'NCR
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>T03-0037 5'NCR
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>T03-0053 5'NCR
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>T03-0078 5'NCR
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>T03-0599 5'NCR
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>T03-0600 5'NCR
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>T03-0634 5'NCR
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>T03-0655 5'NCR
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>T03-1753 5'NCR
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>T03-1808 5'NCR
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>T03-2119 5'NCR (5/2/06)
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>T03-2151 5'NCR
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>T03-2430 5'NCR
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>T03-2431 5'NCR
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>T03-2433 5'NCR
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>T03-2434 5'NCR
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>T03-3194 5'NCR
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>T03-3195 5'NCR
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>T03-3596 5'NCR
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>T03-4111 5'NCR
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>T03-4112 5'NCR
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>T03-4196 5'NCR
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>T03-4311 5'NCR
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>T03-4474 5'NCR
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>T03-4481 5'NCR
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>T04-3552A 5'NCR
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>T04-3552B 5'NCR
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>T04-0424 5'NCR
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>T04-0714 5'NCR
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>T04-0946 5'NCR
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TGTGAAGCCAAATATTGGACAAGGTGTGAAGAGCCGCGTGTGCTCATCTTGA
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>T04-0964 5'NCR
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AGTGTGAAGCCTTAATTTGGACAGGGTGCGAAGAGCCGCGTGTGCTCATCTT
GAGTCCTCCGGCCCCTGAATGCGGCTAACCTTAAACCTGCAGCCATTGTTTGC
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>T04-1004 5'NCR
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GTGAAGCCAATTAGTTGACAGGGTGTGAAGAGCCCCGTGTGCTCATCTTGAT
TCCTCCGGCCCCTGAATGTGGCTAACCTTAAACCTGCAGCCAATGTACACAAT
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>T04-1325 5'NCR
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>T04-1411 5'NCR
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>T04-2387 5'NCR
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>T04-3641 5'NCR
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>T04-3642 5'NCR
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GTGAAGCCATATTTTTGACAAGGTGTGAAGAGCCCCGTGTGCTCACTTTGAGT
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>T04-3643 5'NCR
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ACCCTATGGGTGTGAAGCCATATTTTTGACAAGGTGTGAAGAGCCCCGTGTG
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>T04-3645 5'NCR
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>T04-3903 5'NCR
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>T04-3906 5'NCR
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CCCACTGGTAACAGTGTTCTAGCCTGCGTGGCTGCCTGCACACCCTTTGGGTG
TGAAGCCATATTTTTGACAAGGTGTGAAGAGCCCCGTGTGCTCACTTTGAGTC
CTCCGGCCCCTGAATGTGGCTAACCTTAACCCTGCAGCTAGTGCATGCAATCC
AGCATGTTGCTAGTCGTAACGAGCAATTGCGGGATGGGACCAACTACT

>T04-3934 5'NCR
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AGCATGTTGCTAGTCGTAACGAGCAATTGCGGGATGGGACCAACTACT

>T04-3939 5'NCR
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GTGAAGCCATATTTTTGACAAGGTGTGAAGAGCCCCGTGTGCTCACTTTGAGT
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>T05-1034 5'NCR
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>T05-1430 5'NCR
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>T05-1688 5'NCR
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>T05-1711 5'NCR
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>V06T032267 5'NCR
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TGCTGGGACGCCTTAAGTATGACATGGTGTGAAGACCCGCATGTGCTTAACT
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>V06T04424 5'NCR
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>V05T05509 5'NCR
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>V06T01482 5'NCR
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>V06T0477 5'NCR
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CACGTTACAACGCACTTCCGTTTGCCCCACGAGTACATTC

APPENDIX G. LIST OF ACCESSION NUMBERS FOR THE 5'NON-CODING REGION SEQUENCES OBTAINED FROM GENBANK

AF108179
D00239
X02316
AF542425
AF108185
K02121
L24917
AF542419
AF108180
AF108181
AF108182
AY751783
AF542422
AF542423
AF108183
AF542424
AF108184
AF542426
AF542427
AF108186
AF542429
AF542430
AF542431
AY062273
A10937
AF542432

APPENDIX H. EQUIPMENT USED FOR MICROARRAY

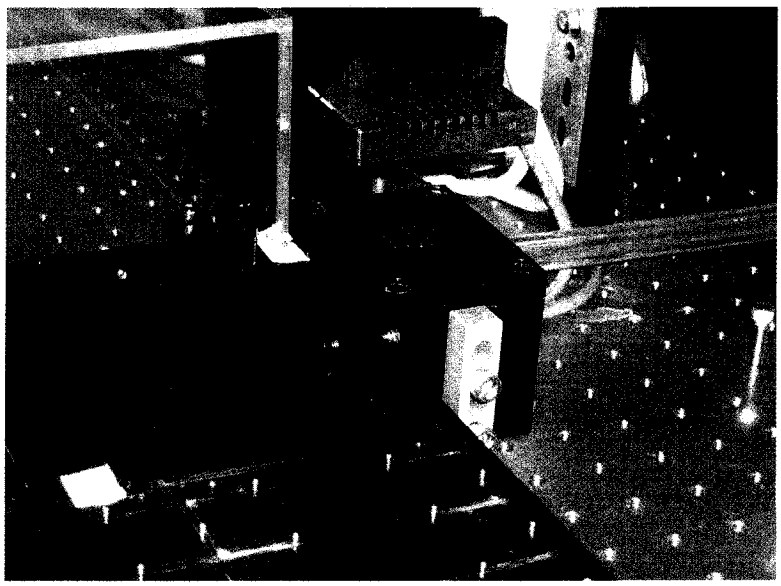

Figure 7. Microarrayer used for spotting PCR products on aminosilane glass slides

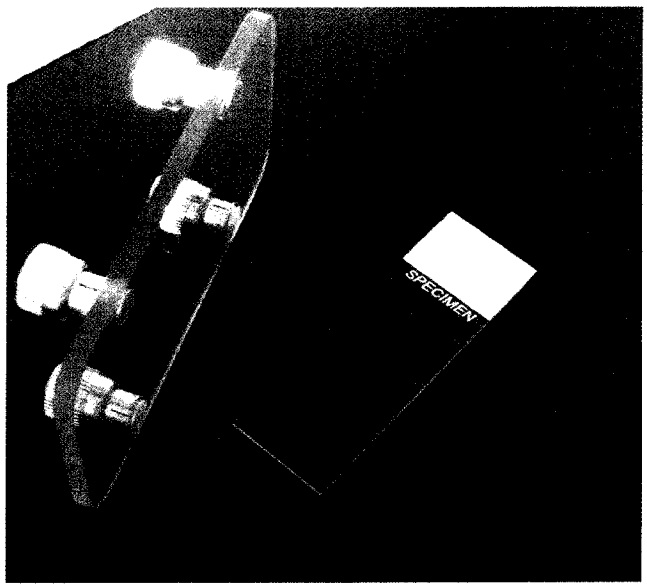

Figure 8. Hybridization chamber with microarray

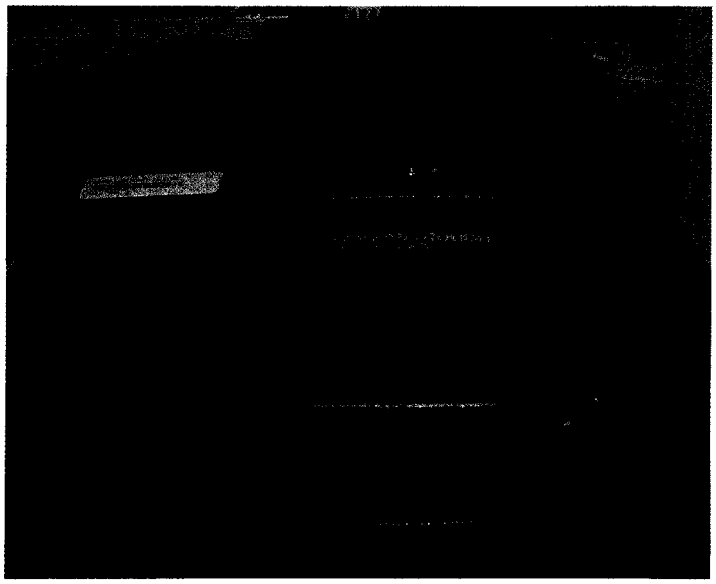

Figure 9. Scanner used for scanning microarrays after hybridization.

APPENDIX I. PCR PRODUCTS SPOTTED ON MICROARRAY

Table 4. Concentration of PCR products spotted on microarray.

Clone ID	OD 260	OD 280	Ratio	[DNA] ng/µl
T32341	0.554	0.303	1.830	27.700
TC68180	0.744	0.407	1.830	37.200
TC67891	0.719	0.398	1.810	35.900
TC1623	0.808	0.449	1.800	40.400
TC50148	0.787	0.413	1.900	39.300
T71919	0.531	0.293	1.810	26.500
TC70632	0.964	0.523	1.840	48.200
TC67686	0.902	0.489	1.840	45.100
T54693	0.799	0.434	1.840	40.000
T00375	0.785	0.430	1.830	39.300
TC67905	1.067	0.589	1.810	53.400
TC67906	1.061	0.567	1.870	53.100
TC67846	0.905	0.484	1.870	45.300
T61921	0.772	0.415	1.860	38.600
T64048	0.623	0.340	1.830	31.200
HRV3	1.588	0.861	1.850	79.400
HRV4	1.833	1.005	1.820	91.700
HRV11	1.776	0.980	1.810	88.800
HRV19	1.666	0.903	1.850	83.300
HRV24	1.818	1.001	1.820	90.900
HRV36	1.741	0.935	1.860	87.000
HRV42	1.607	0.867	1.850	80.300
HRV47	1.497	0.816	1.840	74.900
HRV48	1.821	0.986	1.850	91.100
HRV54	1.677	0.910	1.840	83.900
HRV56	1.483	0.805	1.840	74.200
HRV63	1.423	0.779	1.830	71.200
HRV71	1.566	0.852	1.840	78.300
HRV76	1.763	0.958	1.840	88.100
HRV83	2.117	1.141	1.850	105.800
HRV88	1.594	0.869	1.830	79.700
HRV92	1.676	0.917	1.830	83.800
pUC19	1.068	0.597	1.790	53.400
Blank	0.000	0.000	0.000	0.000

APPENDIX J. MICROARRAY .GAL FILE

"Type=GenePix ArrayList V1.0"

"Block"	"Row"	"Column"	"ID"	"Name"
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1	1	2	T32341	VP4VP2-36
1	1	3	T32341	VP4VP2-36
1	1	4	TC68180	VP4VP2-73
1	1	5	TC68180	VP4VP2-73
1	1	6	TC68180	VP4VP2-73
1	1	7	TC67891	VP4VP2-12
1	1	8	TC67891	VP4VP2-12
1	1	9	TC67891	VP4VP2-12
1	1	10	T21623	VP4VP2-32
1	1	11	T21623	VP4VP2-32
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1	2	1	TC50148	VP4VP2-51
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1	2	5	T71919	VP4VP2-4
1	2	6	T71919	VP4VP2-4
1	2	7	TC70632	VP4VP2-51
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1	2	9	TC70632	VP4VP2-51
1	2	10	TC67686	VP4VP2-19
1	2	11	TC67686	VP4VP2-19
1	2	12	TC67686	VP4VP2-19
1	3	1	T54693	VP4VP2-13
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1	3	3	T54693	VP4VP2-13
1	3	4	T00375	VP4VP2-45
1	3	5	T00375	VP4VP2-45
1	3	6	T00375	VP4VP2-45
1	3	7	TC67905	VP4VP2-10
1	3	8	TC67905	VP4VP2-10
1	3	9	TC67905	VP4VP2-10
1	3	10	TC67906	VP4VP2-8
1	3	11	TC67906	VP4VP2-8
1	3	12	TC67906	VP4VP2-8
1	4	1	TC67846	VP4VP2-4
1	4	2	TC67846	VP4VP2-4
1	4	3	TC67846	VP4VP2-4
1	4	4	TC1921	VP4VP2-1A

1	4	5	TC1921	VP4VP2-1A
1	4	6	TC1921	VP4VP2-1A
1	4	7	T64048	VP4VP2-71
1	4	8	T64048	VP4VP2-71
1	4	9	T64048	VP4VP2-71
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1	4	12	HRV3	5NCR-HRV3
1	5	1	HRV4	5NCR-HRV4
1	5	2	HRV4	5NCR-HRV4
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1	5	11	HRV24	5NCR-HRV24
1	5	12	HRV24	5NCR-HRV24
1	6	1	HRV36	5NCR-HRV36
1	6	2	HRV36	5NCR-HRV36
1	6	3	HRV36	5NCR-HRV36
1	6	4	HRV42	5NCR-HRV42
1	6	5	HRV42	5NCR-HRV42
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1	7	7	HRV63	5NCR-HRV63
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1	7	11	HRV71	5NCR-HRV71
1	7	12	HRV71	5NCR-HRV71

1	8	1	HRV76	5NCR-HRV76
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1	8	3	HRV76	5NCR-HRV76
1	8	4	HRV83	5NCR-HRV83
1	8	5	HRV83	5NCR-HRV83
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1	8	7	HRV88	5NCR-HRV88
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2	2	5	T71919	VP4VP2-4
2	2	6	T71919	VP4VP2-4
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2	4	4	TC1921	VP4VP2-1A
2	4	5	TC1921	VP4VP2-1A
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2	4	8	T64048	VP4VP2-71
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2	6	3	HRV36	5NCR-HRV36
2	6	4	HRV42	5NCR-HRV42

2	6	5	HRV42	5NCR-HRV42
2	6	6	HRV42	5NCR-HRV42
2	6	7	HRV47	5NCR-HRV47
2	6	8	HRV47	5NCR-HRV47
2	6	9	HRV47	5NCR-HRV47
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2	7	9	HRV63	5NCR-HRV63
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2	8	6	HRV83	5NCR-HRV83
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2	9	3	pUC19	pUC19
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2	9	5	Blank	Blank
2	9	6	Blank	Blank
2	9	7	Blank	Blank
2	9	8	Blank	Blank
2	9	9	Blank	Blank
2	9	10	Empty	Empty
2	9	11	Empty	Empty
2	9	12	Empty	Empty

APPENDIX K. EXAMPLE OF MICROARRAY DATA ANALYSES

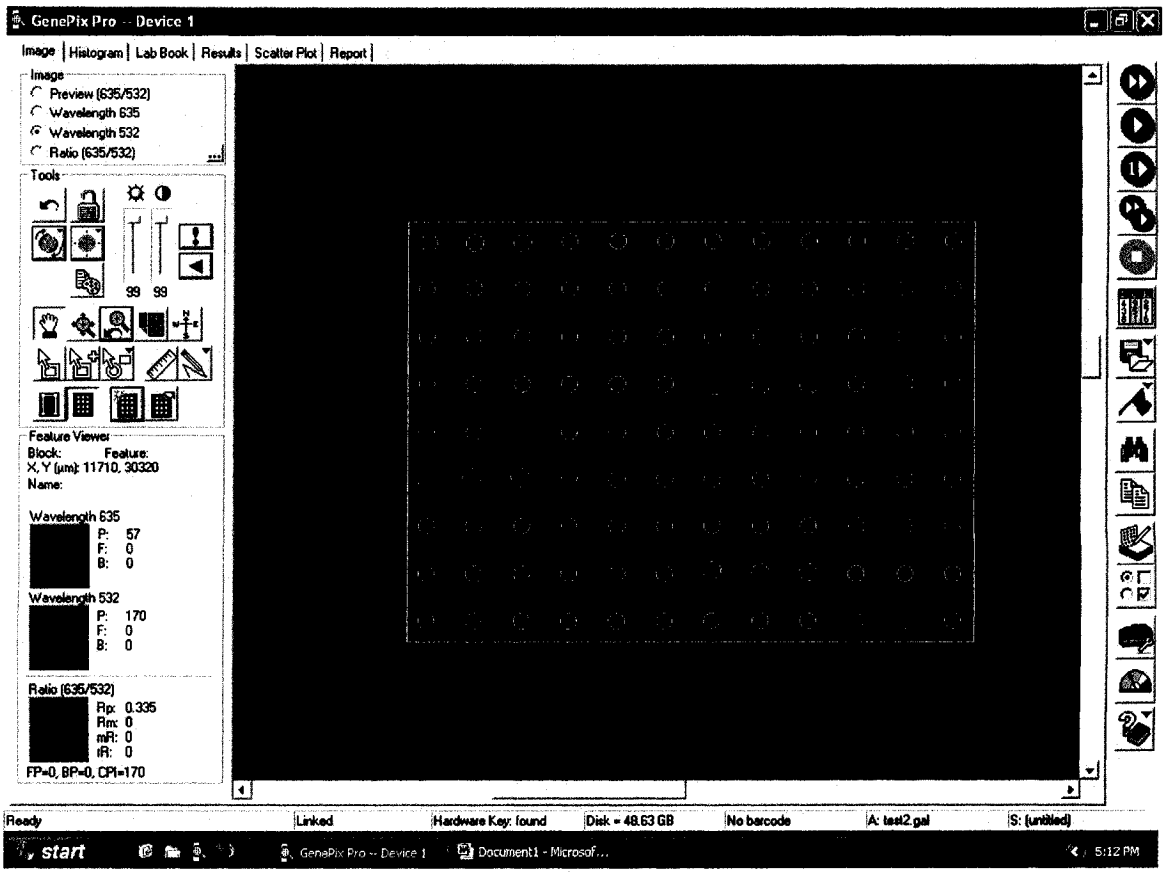

Figure 10. Example of alignment of .GAL file with the top subarray.

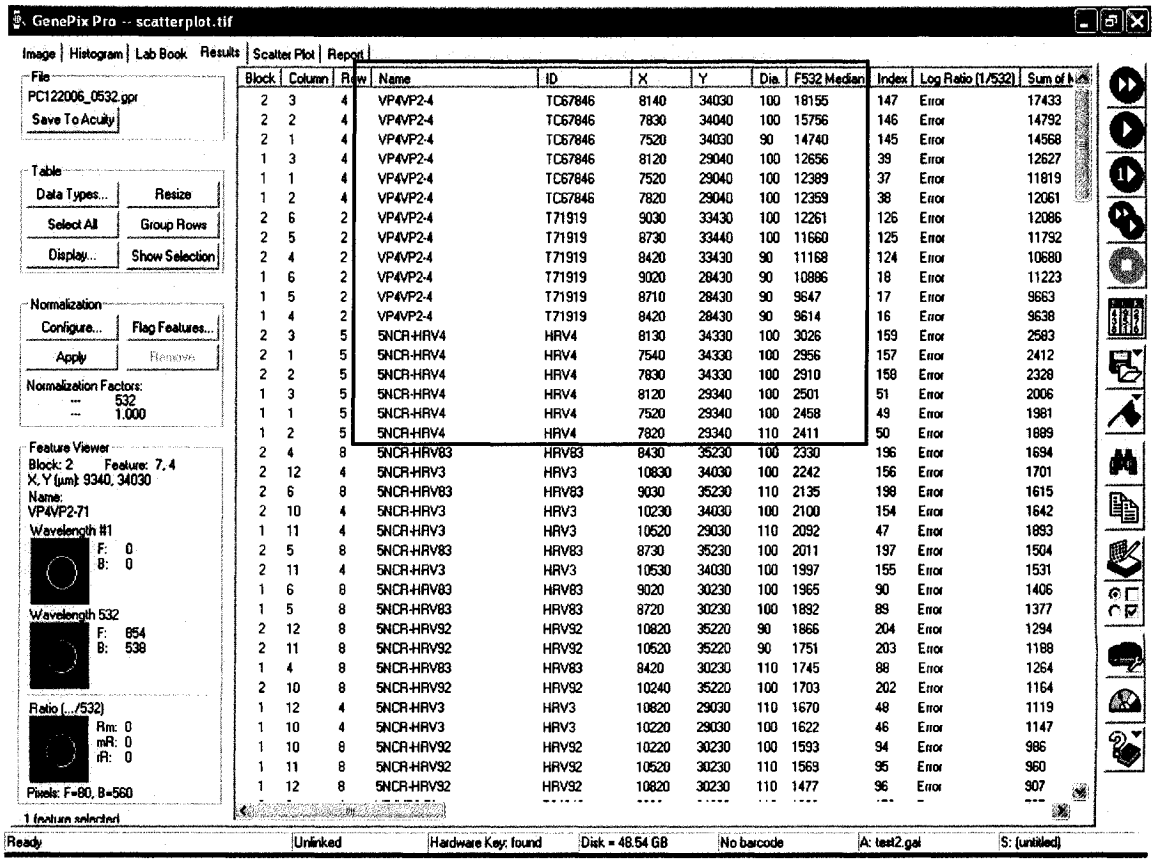

Figure 11. Example of microarray hybridization signals sorted based on highest signal strength.